IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:	) Group Art Unit: 1633
	)
Kyuhyun Lee *et al.*	) Examiner: HIRIYANNA,
	) K. T.
	)
Serial No.: 10/584,383	) Confirmation No.: 3667
	)
Filed: June 26, 2006	)
	)
For: THERAPEUTIC AGENT FOR TREATMENT	)
OF CANCER COMPRISING HUMAN	)
APOLIPOPROTEIN (A) KRINGLES LK68 OR	)
LK8 GENES AS EFFECTIVE INGREDIENT,	)
AND METHOD FOR TREATING CANCER	)
USING THE SAME	)

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

DECLARATION SUBMITTED UNDER 37 C.F.R. §1.132

Sir:

I, Eui-Cheol Jo of MOGAM Biotechnology Research Institute, do hereby declare the following:

1. I am a Research Fellow in the Gene Therapy Laboratory at MOGAM Biotechnology Research Institute.

2. As attested by my curriculum vitae attached hereto, I am knowledgeable and skilled in the field of genetic diagnosis, especially with respect to cancer diagnostic marker.

3. I am a co-inventor in the above-identified application, serial number: 10/584,383.

4. I have read and understand the subject matter of the above-identified application, the Office Action mailed on April 13, 2009, and the claims filed with this Declaration as well as claims as previously presented.

5. The following are my comments and experimental evidence in support of the non-obviousness of the present invention. Unexpectedly superior activity of LK8 and LK68 peptide was seen in preventing migration of endothelial cells when the peptides were expressed from Adenovirus and AAV, compared with those produced from *E. coli*. Over a one-hundred fold greater activity was seen than those produced from *E. coli*, which is much higher than what one would have expected even taking into account glycosylation and other post-translational modifications that are available only through mammalian cell processing. Our experimental results show that the enhanced activity is not the result of mere glycosylation or known post translational modifications.

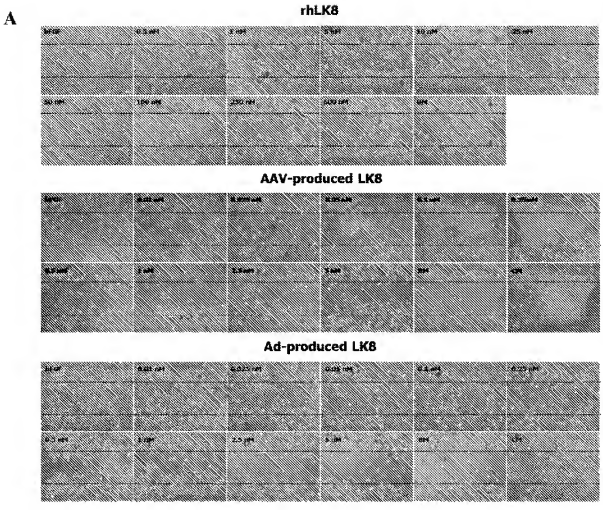
Example 1: Post-translational modification of LK8 and LK68

We compared inhibitory effects against human vascular endothelial cell of LK8 and LK68 polypeptides isolated from *E. coli* and gene carriers comprising polynucleotide encoding LK8 and LK68 of the present invention and found that the gene carriers showed more than one-hundred (100) fold activity compared with the *E. coli* produced polypeptides. The half maximal inhibitory concentration (IC50) of LK8 and LK68 was measured using wound migration assay and confirmed using transwell migration assay (Boyden chamber assay).

The wound migration assay demonstrated that LK8 and LK68 peptides suppressed migration of bFGF-stimulated endothelial cell in a dose-dependent manner (See Figs. 1 and 2). IC50 of LK8 and LK68 peptides produced by AAV and Ad were equally 0.25 nM, while those of the *E. coli*-derived peptides were about 25 nM and 50 nM, respectively (See Figs. 1 and 2).

Using a more sensitive transwell migration assay, we confirmed that LK8 peptide suppressed migration of VEGF-stimulated endothelial cell in a dose-dependent manner (See Fig. 3). 0.05 nM adenovirus-produced LK8 peptide exerted almost equivalent inhibitory activity to that reached with 100 nM LK8 peptide from *E. coli*. IC50 of adenovirus-produced LK8 peptide was determined to be 0.025 nM, as compared with about 50 nM with the LK8 peptide from *E. coli*. (See Fig. 3). Consequently, Adenovirus-produced LK8 and LK68 peptides inhibited VEGF and FGF-stimulated endothelial cell migration ***in vitro*** by more than 80% ($P<0.01$ versus the negative control group), which was 100 fold superior to the activity exerted by the same molecules derived from recombinant microbes. These results suggest great advantage of administering gene carrier directly into patients, rather than frequent injection of a larger amount of protein drug. To delineate the reason for this difference, we set out to analyze physico-chemical properties of LK8 and LK68 produced by different methods.

FIG. 1



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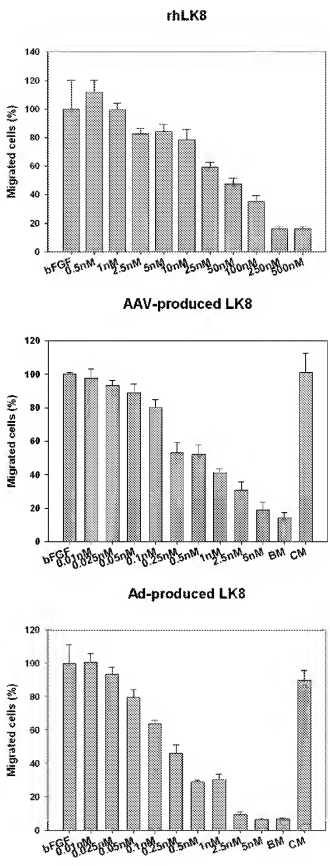
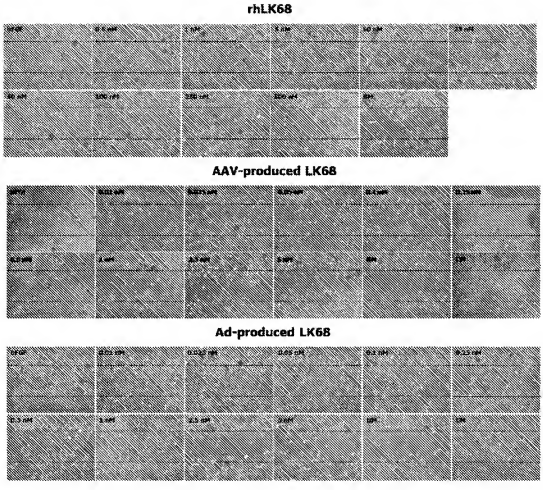

rhLK8
Migrated cells (%)
140
120
100
80
60
40
20
0
bFGF
0.5nM
1nM
2.5nM
5nM
10nM
25nM
50nM
100nM
250nM
500nM
AAV-produced LK8
Migrated cells (%)
120
100
80
60
40
20
0
bFGF
0.01nM
0.025nM
0.05nM
0.1nM
0.25nM
0.5nM
1nM
2.5nM
5nM
BM
CM
Ad-produced LK8
Migrated cells (%)
120
100
80
60
40
20
0
bFGF
0.01nM
0.025nM
0.05nM
0.1nM
0.25nM
0.5nM
1nM
2.5nM
5nM
BM
CM

Fig. 1. Wound migration assay for comparison of LK8 activity. Confluent HUVECs were wounded by scraping with a 2 μL pipette tip, which denuded a monolayer strip of 300 μm in width. They were incubated with purified rhLK8, adeno-associated virally produced LK8 or adenovirally produced LK8. Control cultures were incubated in EBM-2 medium (Clonetics) with or without the addition of bFGF (3 ng/mL; Roche, Mannheim, Germany). The rate of wound closure was observed over the next 8 h. Cells that migrated into the denuded area were pictured (A) and counted. The cells in denuded area were counted and expressed as percentage of migrated cells (B).

FIG. 2



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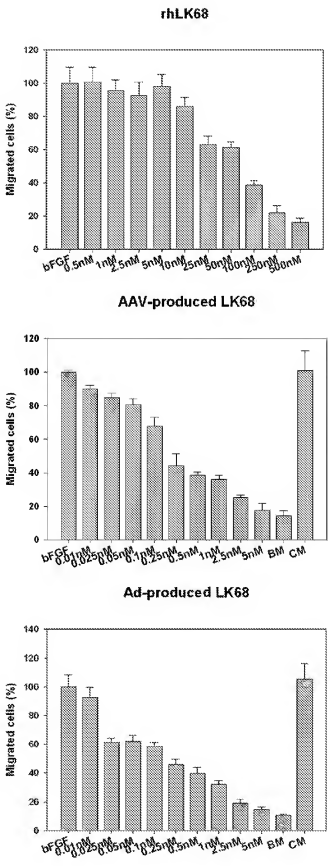
rhLK68
Migrated cells (%)
120
100
80
60
40
20
0
bFGF 0.5nM 1nM 2.5nM 5nM 10nM 25nM 50nM 100nM 250nM 500nM
AAV-produced LK68
Migrated cells (%)
120
100
80
60
40
20
0
bFGF 0.01nM 0.025nM 0.05nM 0.1nM 0.25nM 0.5nM 1nM 2.5nM 5nM BM CM
Ad-produced LK68
Migrated cells (%)
140
120
100
80
60
40
20
0
bFGF 0.01nM 0.025nM 0.05nM 0.1nM 0.25nM 0.5nM 1nM 2.5nM 5nM BM CM

Fig. 2. Wound migration assay for comparison of LK68 activity. All procedures were same as described in Fig. 1. Instead of LK8, Denuded HUVECs were incubated with purified rhLK68, adeno-associated virally produced LK68 or adenovirally produced LK68. Cells that migrated into the denuded area were pictured (A) and counted. The cells in denuded area were counted and expressed as percentage of migrated cells (B).

FIG. 3

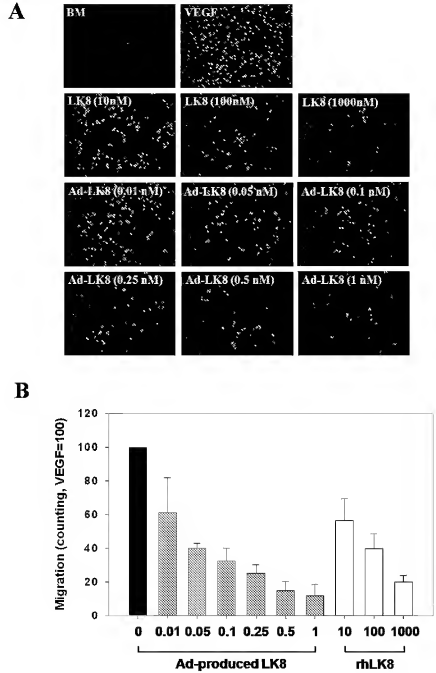

Fig. 3. Transwell migration assay for comparison of LK8 activity. Confluent HUVEC monolayers were cultured with media lacking growth supplement for overnight prior to harvest. Harvested cells were suspended at 10^5 cells into fibronectin precoated transwell insert (8-μm pore; Costar). HUVEC migration was stimulated by the addition of VEGF (10ng/ml) to the

lower chamber and suppressed by the addition of purified rhLK8 or Adenovirus-produced LK8 proteins to the upper chamber. After 5 h, migrated cells were stained (A) and counted (B).

One clear way to measure the difference in activities between a polypeptide isolated from *E. coli* and a polypeptide expressed by gene injection is to determine their molecular weights. Since a polypeptide expressed by gene injection is modified post-translationally via endoplasmic reticulum and Golgi complex generally, the polypeptide tends to have larger molecular weight than a polypeptide isolated from *E. coli*. Although the molecular weights of LK8 and LK68 polypeptides are calculated from their cDNA sequences to be 9 kDa and 35 kDa, respectively, proteins expressed from human cell line have larger molecular weights, 14 kDa and 55 kDa, respectively (See Fig. 4).

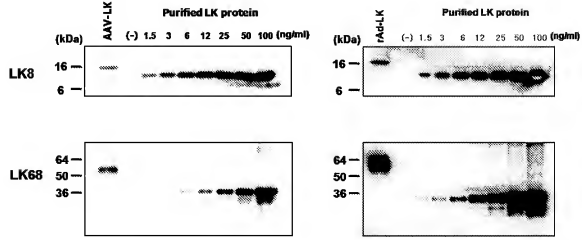

Fig. 4. Molecular weight difference between purified recombinant human LK (rhLK) and adenovirus-produced LK (rAd-LK). Western blot analysis was conducted using a rabbit anti-LK8 antibody.

We hypothesized that since translational modification affects LK8 and LK68 molecular weight to a large extent, such modification would also influence protein activity. The following experiments were carried out in order to confirm the correlation between post-translational

modification, molecular weight change and protein activity.

Example 2: Investigation of post-translational modification

Post-translational modification includes N-, O-Glycosylation, phosphorylation, lipidation such as acylation, myristoylation, palmitoylation, farnesylation and SUMOylation, and so forth. Secreted proteins like LK8 and LK68 tend to be glycosylated. Therefore, we performed the following experiment in order to investigate whether the reason for the huge gain in activity was due to glycosylation.

2-1: Identification of N-Glycosylation and O-Glycosylation

In order to identify whether the glycosylation is N-type or O-type, we treated human cell lines expressing LK68 and LK8 with tunicamycin (an N-glycosylation inhibitor) and GalNAc-α-O-Benzyl (an O-glycosylation inhibitor). Secretion of LK68 is inhibited by treatment with N-glycosylation inhibitor. However, secretion of LK8 is not affected (See Fig. 5).

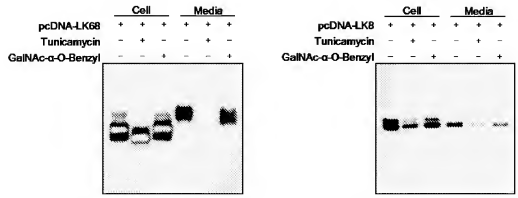

Fig. 5. Western blot analysis of LK8 and LK68 proteins isolated from mammalian cells transfected with plasmids comprising LK8 and LK68 gene, respectively in the presence or absence of tunicamycin and/or GalNAc-α-)-Benzyl.

Since we confirmed that LK68 is N-glycosylated, we investigated the extent of N-

glycosylation of LK68 by treating the protein with PNGaseF which cleaves N-glycosyl group from LK68 protein. As a positive control, we treated Rituxan® known to have a single glycosylation site with PNGaseF (See Fig. 6).

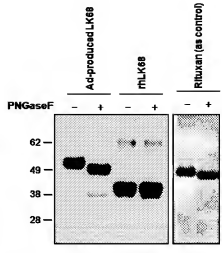

Fig. 6. Western blot analysis of LK68 proteins produced from Adenoviral infected mammalian cells and recombinant LK68 protein produced from *E. coli* treated or not treated with PNGaseF (an N-glycosylase).

LK8 protein exhibited no size difference when treated with PNGaseF (LK8 has no N-glycosylation site). LK68 protein has a single N-glycosylation site, and its gel mobility was compared with band shift of Rituxan®. Thus, we concluded that N-glycosylation cannot account for all of the differences in size and activity of the LK8 and LK68 proteins generated from mammalian cell.

2-2) Identification of other posttranslational modification

We assumed that there are other modifications specific to LK8 and LK68. Applying a post-translational analysis tool ExPASy program to LK8 and LK68, one SUMOylation site was

discovered in LK68. We treated the proteins with SUMOylase to remove possible SUMO proteins bound to LK68. However, no difference in molecular weight was found after treatment (See Fig. 7). At present, we do not know what kind of post-translational modification LK68 has. However, we believe that the modification is not a generally known post-translational modification judging from the experimental evidence provided here.

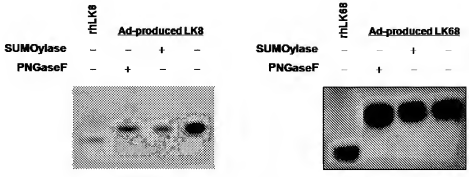

Fig. 7. Western blot analysis of LK8 and LK68 produced by Adenoviral infected mammalian cells and LK8 and LK68 produced by *E. coli* in the presence of absence of SUMOylase and/or PNGaseF.

Consequentially, we believe that LK8 and LK68 proteins undergo non-conventional post translational modifications in addition to glycosylation for LK68. Such high activities of LK68 and LK8 proteins are not expected because these proteins are assumed to have unique post-translational modification.

6. The undersigned hereby declares that all statements made herein based upon knowledge are true, and that all statements made based upon information and belief are believed to be true; and further, that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C. §1001, and that such willful false statements may jeopardize the validity of the application or any patent

issued thereon.

DATED: _____ _____

Name: Eui-Cheol Jo